Chapter 4

The Function of Rab35 in Development and Disease

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Abstract

Rab35 mediates membrane trafficking between the plasma membrane and the early endosomes at the cell surface. Our understanding of the cellular function of Rab35 reveals its role in development and diseases. In the developmental context, Rab35 has been shown to play an important role in regulating epithelial polarity, lumen opening, myoblast fusion, intercalation of epithelium, myelination, neurite outgrowth, and oocyte meiotic maturation. Disruption of recycling endosome mediated by Rab35 has been linked to several neurological diseases, including Parkinson's disease and Down syndrome. In addition, because Rab35 modulates cell migration through its interaction with various effectors, Rab35 plays an important role in cancers. Lastly, the Rab35-mediated recycling endosomal pathway and exocytosis is utilized by pathogens or hijacked by pathogens to promote their infection and survival. This review summarizes the function of Rab35 in endocytosis and focuses on the role of Rab35 in the context of development and diseases.

Keywords: small G proteins, Arf6, development, cell migration, protein trafficking

1. Introduction

Rab proteins constitute the largest subset of Ras-family small guanosine triphosphates (GTPases). Over 60 mammalian Rab proteins have been identified [1]. Rab35 is an evolutionarily conserved unique Rab GTPase that mediates membrane trafficking between the plasma membrane and endosomes in eukaryotic cells [1]. Similar to all G proteins, Rab35 undergoes molecular switching from active GTP-bound state to inactive GDP-bound state. The activity of Rab35 is highly regulated with four different Rab35 guanine-nucleotide exchange factor (GEF) and five Rab35 GTPase-activating proteins (GAPs). Depending on which effector proteins are associated with Rab35-GTP, it can mediate many cellular functions, such as cytokinesis, phagocytosis, cell migration, and exosome release [2–7]. Most of its cellular functions

involve the regulation of Rab35 in actin polymerization, Arf6 inactivation, or phosphoinositides (PtdIns(4,5)P2) [2, 8–10]. This review briefly summarizes the regulation of Rab35 in endocytosis and focuses on Rab35 in the context of development and diseases.

2. Rab35 mediates protein trafficking

Small G proteins mediate endosomal trafficking and maintain cell surface homeostasis. Two major types of endocytosis are the clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) [11]. CME involves the selective uptake of plasma membrane that is dependent on dynamin for vesicle scission. CIE is dynamin-independent and depends on free cholesterol at the plasma membrane; CIE does not require specific endocytic sorting sequence and is known as the bulk endocytic process [12]. The homeostasis at the cell surface requires balanced CME and CIE and the coordinated regulation by Rab35 and Arf6 [9]. Rab35 and Arf6 work antagonistically at the plasma membrane where Arf6 recruits the Rab35 GAP to inactivate Rab35, and Rab35 recruits the Arf6 GAP to inactivate Arf6. Thus, Rab35 from CME and the Arf6 from CIE work together to balance the two branches of the endocytic pathway [11].

Following endocytosis, endocytic vesicles converge on early endosomes where the cargos are sorted to be recycled or transported to late endosomes before eventually fusing with lysosomes. Activated Rab35 recruits effectors that mediate the formation of recycling tubules or vesicles. A diverse array of cargoes have been reported to undergo Rab35-dependent endocytic recycling back to the plasma membrane [13]. Overall, Rab35 plays a conserved role in mediating endocytosis recycling after cargo internalization.

The function of Rab35 is dependent on the effector proteins that bind to the active Rab35-GTP. Several key effectors of Rab35 have been identified that reveal its cellular functions. The level of the PtdIns(4,5)P2 lipid on endosomes is mediated by Rab35, since it binds to PtdIns(4,5)P2 phosphatase, OCRL [2]. Depletion of either Rab35 or OCRL leads to accumulation of PtdIns(4,5)P2 and F-actin binding proteins in enlarged peripheral endosome. Thus, Rab35 functions with OCRL to hydrolyze PIdIns(4,5)P2 on new endosomes and help define the lipid identity of early endosomes.

Fascin is another Rab35 effector protein. It crosslinks actin and assembles F-actin filaments into parallel bundles. Rab35-GTP recruits fascin in regulating *Drosophila* bristle development (Section 3.3) [10]. The shape and growth of the bristles depend on actin bundles. Rab35 also induces actin-rich protrusions in PC12 cells and regulates lamellipodia and filopodia formation in *Drosophila* [4].

Rab35 and Arf6 have been found to function antagonistically in regulating membrane trafficking [14]. Two Rab35 effectors are MICAL-L1 and ACAP2 (Arf6 GAP) that are involved in neurite outgrowth (Section 3.9.3) [15]. Another effector protein that interacts with Rab35-GTP in regulating neurite outgrowth is RUSC2. The overexpression of the RUN domain of RUSC2 inhibits Rab35-iduced neurite outgrowth in PC12 cells [16]. The role of RUSC2 downstream of Rab35 is not known. It is likely that the Rab35 interactome is far from complete.

3. Rab35 in development

The function of Rab35 in development has been examined mainly in the fly and worm. Various cell type such as myoblasts, fly S2 cells, oocytes, osteoclasts, neurons and oligodendrocytes have been used to study specific developmental processes. This section summarizes our current understanding of the role of Rab35 in the context of development.

3.1. Rab35 polarizes the fly tracheal seamless tubes

Drosophila trachea consists of multicellular, autocellular, and seamless tubes [17]. How seamless tubes are constructed is not well known; however, vesicular transport of apical membrane is thought to play an important role in forming the seamless tubes. During tubulogenesis, tip cells undergo epithelial to mesenchymal transition and initiate branching morphogenesis. A subpopulation of tip cells then differentiate in to terminal cells. A single seamless tube forms within each branched extension of the terminal cell. Together with Rab35, Whacked (a Rab35-GAP) mediates polarization of the growth of seamless tubes. Constitutive activation of Rab35 resulted in overgrowth of tubes at the terminal cell branch tips [18]. Conversely, Rab35-S22N dominant negative (DN) leads to ectopic branching surrounding the terminal cell nucleus. The function of Whacked and Rab35 is not in actin regulation, but in transporting vesicles from a recycling endosome to the branch tips [18]. Further, the polarized vesicular trafficking mediated by Rab35 is dependent on dynein motor-mediated transport towards the apical membrane [18]. Thus, polarized growth of the seamless tubes involves coordination of Rab-GAP, Rab35, and dynein.

3.2. Rab35 regulates epithelial organ lumen opening formation

Epithelial organs such as the lungs and kidneys are composed of a polarized cell monolayer surrounding a lumen. Madin-Darby canine kidney (MDCK) cells have been used as a model to examine the establishment of epithelial polarity and lumen [19]. It is thought that new apico-basal polarity in cysts arises from divisions of a single cell where apical transmembrane proteins are transcytosed from the plasma membrane to the cell-cell contact site. A recent study using MDCK cells indicated that Rab35 was found to directly interact with Podocalyxin (PODXL), a classical apical marker that has anti-adhesive properties that promote cell-cell repulsion at the apical membrane [20]. Rab35 knockdown with RNAi resulted in a complete inversion of polarity so that the PODXL localizes on the membrane facing the extracellular matrix instead of the lumen [20]. Rab35 establishes the apico-basal polarity by transporting PODXL to the site of lumen formation [20].

3.3. Rab35 recruits fascin to form *Drosophila* bristles

The *Drosophila* bristle is a mechanosensory organ where their shape and growth depends on actin bundles as the important structural component. *Drosophila* Rab GTPases were systematically tested for their impact on development with the use of DN mutant proteins. Of these 31 fly Rab GTPases tested, Rab35-S22N DN and Rab35 RNAi in the peripheral nervous system resulted in bristle morphology defects [10]. Adult bristles in reduced Rab35 function

were structurally abnormal, with sharp bends and kinks. Rab35-GTP binds to fascin, an actin cross-linking protein that organizes F-actin into bundles. In Rab35 DN cells, actin is found to be loose and disconnected. Although Rab35 has no effect on actin bundling *in vitro*, its association with fascin allows it to control when or where actin is bundled *in vivo* [10]. It was proposed that Rab35 may recruit fascin to initiate the cytoplasmic extension required for bristle extension. Insufficient Rab35 function would lead to bends and kinks in the bristles. Thus, Rab35 recruits fascin to subcellular location to drive actin bundling at the leading edge of cell protrusions to form the *Drosophila* bristles.

3.4. Rab35 functions in *Drosophila* germband extension

During development, cell assemblies that involve coordination of cellular adhesion and shape changes are needed to form tissues and organs. Internal organs such as the palate cochlea, gut and the kidney all require tissue elongation to shape an elongated body axis of an developing animal [21]. Cellular reshaping during organ formation requires the function of apical and junctional cytoskeletal and adhesion proteins. During *Drosophila* germband extension (GBE), cells undergo coordinated planar contraction of interfaces between Anterior-Posterior (AP) of neighboring cells. In the fly epithelium, Rab35 is expressed at low levels in the plasma membrane at the AP interfaces during active interface contractions. During this time, Rab35 functions in a conserved cell-shaping mechanism in which Rab35 shrinks AP cell surfaces by feeding endocytosed membranes and protein components to early endosomes to decrease plasma membranes to shape the epithelial cells. Rab35 directs the progressive shortening of anterior interface in response to apical area oscillations [22]. In addition, another developmental context where Rab35 shapes the epithelium is during mesodermal invagination where a ventral furrow is formed. In Rab35 knockdown embryos, rates of apical construction are greatly reduced and the embryo failed to form a ventral furrow for mesodermal invagination [22]. Thus, Rab35 functions by shrinking cell surfaces in shaping epithelial cell behaviors during development.

3.5. Rab35 regulates mouse oocyte meiosis

During meiosis, the oocytes undergo nuclear and cytoplasmic maturation where the migration of intracellular components such as spindle, mitochondria, and cortical granules is important for subsequent embryonic development. Rab35 localizes in the ooplasm at the germinal vesicle (GV) stage [23]. After germinal vesicle breakdown (GVBD), Rab35 is distributed at the spindle and colocalizes with α-tubulin. Rab35 RNAi treated oocytes displayed abnormal spindle morphology with multiple poles of spindle components, indicating that Rab35 regulates mouse oocyte spindle formation [23]. In addition, Rab35 RNAi and antibody blocking experiments indicated that GVBD is not affected, but polar body extrusion defect was observed. Overall, Rab35 was found to be important for forming spindles of oocytes during oocyte meiotic maturation and activation [23].

3.6. Terminal steps of cytokinesis is regulated by Rab35

Intracellular transport is essential for animal cytokinesis, with both the secretory and the endocytic pathways being implicated in the late phase of cytokinesis. Previously Rabs important in cytokinesis was screened with RNAi in S2 cells, in search for binucleated cells that

failed to undergo cytokinesis [2]. Rab35 is found to be required for the stabilization of the cytokinesis bridge connecting the daughter cells after furrow ingression as well as the abscission [2]. The proposed mechanism of Rab35 here is that Rab35 controls the localization of phosphatidylinositol 4,5-bis phosphate (PIP2) and SEPT2 at the bridge which are required for the stability of cytokinesis completion [2, 24, 25]. Rab35-mediated endocytic recycling is important for the stabilization of the late stage cytokinesis and abscission.

3.7. Rab35 regulates endocytic recycling of yolk receptor

Previously genetic mutants of endocytosis were identified in *C. elegans*. In rme-4 and rme-5 (Rab35) mutants, yolk uptake was greatly reduced [26]. Yolk uptake is necessary to support early embryonic growth. Biochemical and genetic evidence indicate that RME-4 recruits or activates Rab35 on endocytic vesicles to recycle receptors to the plasma membrane. RME-4 contained DENN domain that interacts with Rab35-GDP form, not the Rab35-GTP or WT forms [26]. In rem-4 and rem-5 mutants, sorting and/or recycling of yolk receptor RME-2 was impaired. Interestingly, in *C. elegans*, rab35 null mutants are viable and fertile, so it is not essential for the abscission step of cytokinesis as previously reported [2]. RME-4 is generally required for endocytosis in multiple cell types, whereas Rab35 may be cell type or cargo-specific.

3.8. Rab35 mediates myoblast fusion

During embryonic development, assembly and disassembly of cadherins play an important role in morphogenesis, cell differentiation, growth and migration [27]. Rab35 regulates cadherin trafficking and stabilization at cell-to-cell contacts to mediate myoblast fusion [28]. Rab35-S22N DN and RNAi results indicated a reduction of N- and M-cadherin at cell-to-cell contacts and increased accumulation in intracellular vacuoles. Rab35 RNAi and DN inhibited myoblast differentiation by preventing myoblast fusion to form myoblasts [28]. Overexpression of Rab35-WT and constitutively active Rab35-Q67L indicated their colocalization at the plasma membrane with $PI(4,5)P_2$, but no perturbation of $PI(4,5)P_2$ was observed [28]. The proposed mechanism is that Rab35 function is required for $PI(4,5)P_2$ production which stabilizes cadherin at cell-cell contact sites. Taken together, these results indicate that Rab35 regulates cadherin-dependent adherens junction formation and myoblast fusion [28].

3.9. Rab35 in the nervous system

3.9.1. Rab35 suppresses oligodendrocyte differentiation

During development of the central nervous system, oligodendrocytes precursor cells undergo cell division and migrate along axons where oligodendrocytes differentiate to wrap axons with myelin sheaths [29]. The dynamic morphological changes are in part mediated by small GTPase signaling. The regulatory role of Rab35 in oligodendrocyte differentiation was examined in FBD-102b (mouse oligodendroglial cells) cells [30]. Rab35 activates its effector protein ACAP2 (a Arf6-GAP) to deactivate Arf6, which inhibits FBD-102b differentiation [30]. Consistent with this result, knockdown of Arf6 with RNAi inhibits oligodendrocyte differentiation. In oligodendrocyte and neuronal cocultures, knockdown of Rab35 or ACAP2 promotes myelination, and inhibition of cytohesin-2 (Arf6-GAP) or Arf6 knockdown inhibits myelination [30]. These

studies revealed that Rab35 and Arf6 function antagonistically in regulating the differentiation of oligodendrocyte and myelination.

3.9.2. Rab35 coordinates synaptic vesicle trafficking and turnover

At least 30 out of the 60 mammalian Rab GTPases are associated with synaptic vesicle (SV) pools [31]. Antagonistic and synergistic functions of molecules within the Rab35 and Arf6 signaling network are necessary for regulating SV protein trafficking, degradation, and neurotransmitter release. Depending on neuronal activity, SVs may either get exocytosed when Arf6 is activated, or SVs can get recruited to presynaptic endosomes when Rab35 is activated [32]. Dysfunction of this signaling network may induce neurologic and neurodegenerative diseases. The molecular mechanism of SV protein turnover was further defined by using the rat hippocampal neurons [33]. Rab35 degrades SV proteins via the endosomal sorting complex required for transport (ESCRT) pathway, by recruiting Rab35 effector and ESCRT protein, Hrs. Upon neuronal stimulation, ESCRT proteins are recruited to SV pools to degrade specific SV proteins [33].

In addition, Rab35 and its GAP, Skywalker (Sky), were found to be key players in the endosomal sorting/recycling of SV proteins [34]. Sky was identified to facilitate endosomal trafficking of synaptic vesicles at *Drosophila* neuromuscular junction boutons, by controlling Rab35 GTPase activity. *Sky* mutants harbor a larger releasable pool of synaptic vesicles and led to increased basal neurotransmitter release. Rab35 DN rescues Rab35 GAP *sky* mutant phenotypes [33]. Consistent with the antagonistic function of Rab35 and Arf6, Arf6 loss-of-function is similar to that observed in *sky* mutants (result in accumulation of Rab35-GTP). Thus, Rab35 and Arf6 antagonistic regulation of synaptic endosomal trafficking maintains the SV protein homeostasis.

3.9.3. Rab35 mediates neurite outgrowth

Rab35 has been shown to promote neurite outgrowth of PC12 cells in response to nerve growth factor (NGF) stimulation [35, 36]. Upon nerve growth factor (NGF) stimulation, Rab35 accumulates in Arf6-positive endosomes [36]. Both Rab35 and Arf6 work antagonistically to regulate neurite outgrowth. The same Rab35 effector, ACAP2 (or centaurin-β2) that regulates the differentiation of oligodendrocytes (Section 3.9.1), is recruited to the Arf6-positive endosomes in a Rab-35-dependent manner upon NGF stimulation. The Arf6-GAP activity of ACAP2, leading to Arf6 inactivation, was required for NGF-induced neurite outgrowth [36]. In addition, Rab35 was found to form a tripartite structure with MICAL-L1 and ACAP2 and recruit them to Arf6-positive endosomes in response to NGF. MICAL-L1 and ACAP2 cooperatively recruit EHD1, which belongs to the dynamin-like C-terminal Eps15 homology domain protein family [37]. EHD1 promotes membrane trafficking of various receptors, mainly from recycling endosomes to the plasma membrane. EHD1 functions as molecular scissors that facilitate fission of vesicles from recycling endosomes via its ATPase activity. Knockdown of Rab35, MICAL-L1, ACAP2, and EHD1 all resulted in shortened neurite outgrowth, indicating the importance of each of these components [36, 38]. In summary, Rab35 recruits and coordinates MICAL-L1 and ACAP2 to Arf6-positive endosomes. At the same time, EHD1 is recruited by binding to MICAL-L1 where it may facilitate neurite tip outward growth by mediating fission of vesicles that target to neurite tips from recycling endosomes during neurite outgrowth.

Rab35 has been proposed to act as a master Rab that determines the intracellular localization of MICAL-L1, which functions as a scaffold for other recruited Rabs [15]. Upon NGF stimulation, Rab35 localizes to Arf6-positive recycling endosomes and recruits MICAL-L1, which interacts with Rabs 8, 13, and 36 [15]. Each of these recruited Rabs functions in a non-redundant manner downstream of Rab35 and MICAL-L1 in regulating neurite outgrowth [15]. Knockdown of individual MICAL-L1 interacting Rabs did not alter MICAL-L1 localization but inhibited NGF-induced neurite outgrowth. Overall, the NGF stimulation activates Rab35 which recruits several other Rabs at recycling endosomes that supply membranes and proteins to enable neurite outgrowth.

3.9.4. Rab35 functions in axon elongation

Neurons acquire an asymmetric morphology during embryonic development to establish neuronal polarization, where a single axon and several dendrites are formed [39]. Neuronal polarized trafficking is dependent on the supply of membrane needed to cell expansion and the differential distribution of proteins. This process involves Rab35 and its regulators. Rab35 was found to function in axon elongation that is regulated by p53-related protein kinase, or PRPK [40]. PRPK is a negative regulator of Rab35 that promotes the degradation of Rab35 via the ubiquitin proteasome degradation pathway. Another protein, microtubule-associated protein 1B (MAP1B), interacts with PRPK to inhibit its degradation of Rab35 [40]. MAP1B is necessary for proper axon outgrowth, as decreased MAP1B expression reduces axon length. MAP1B knock out is rescued by Rab35 overexpression or PRPK inactivation. Neurons overexpressing Rab35 WT and active Rab35-Q67L exhibited a significant increase in exon length. In contrast, Rab35-S22N DN transfected neurons had reduced axon length. In addition, Rab35 activates Cdc42 by either direct activation of Cdc42 or transporting vesicles containing polarity determinants to the elongating exons [40]. Overall, these results indicate that Rab35 is critical for mediating neuronal polarization trafficking to elongate axons.

4. Rab35 in diseases

Disruption of recycling endosome mediated by Rab35 has been linked to several neurological diseases, including Parkinson's disease and Down syndrome [41, 42]. In addition, because Rab35 modulates cell migration through its interaction with Wnt/Dvl signaling pathway and F-actin modulators, Rab35 plays an important role in cancers (see Section 4.3). Lastly, the Rab35-mediated recycling endosomal and exocytosis pathways are used by pathogens to promote their infections and survival (see Section 4.4). This section summarizes the role of Rab35 in various diseases (**Table 1**).

4.1. Rab35 may be involved in Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disease in which the patient's dopaminergic neurons in the substantia nigra are impaired [43]. Lewy bodies composed of abnormal α-synuclein accumulate in substantia nigra neurons of PD patients [44]. The serum levels of Rab35 was high in PD patients and in the substantia nigra of mice models for PD [41]. Overexpression

Disease	Function	Potential molecular mechanism	Ref
Parkinson's disease	Endocyclic recycling of α-synuclein	Unknown	[41]
Down syndrome	Exosome release	Unknown	[42]
Breast cancer	Promotes cell migration	Activation of Rac1 via Wnt5a/Dvl2.	[5, 43]
		Active Rab35 and MICAL1 generate ROS and activate Akt pathway	
Lung cancer	Enhance cell polarization and migration	Rab35 mediates interaction of RUSC2 and GIT2; mediate GIT2 phosphorylation	[44]
Allergy-induced asthma	Delayed TCR recycling; increase cytokines	DENND1B interacts with AP-2 to mediate Rab35 GTP exchange	[45]
Amoebic colitis *Entamoeba histolytica*	Uses Rab35 to phagocytose RBCs	Unknown	[46]
Uropathogenic *E. coli*	Iron acquisition; lysosome evasion	Exploits host TfR1 to acquire iron	[47]
Enterohemorrhagic *E. coli*	Inhibit host endocyclic recycling pathway	Bacteria EspG interacts with Arf6-GTP	[48]
Legionnaires disease	Evade fusion with host lysosomes	LepB stimulates GTP hydrolysis on Rab35	[49, 50]
		AnkX modifies Rab35 with phosphocholine	[51]
Antrax *Bacillus anthracis*	Endocytosis and release anthrax lethal toxin	Rab35 mediates MAPKK cleavage and exosome formation	[7]

Table 1. Summary of Rab35 in diseases.

of Rab35 in SH-SY5Y cells (cell line model to study neuronal function) resulted in increased aggregation and secretion of α-synuclein [41]. Although no detailed mechanism of Rab35 in the pathogenesis of PD is known, Rab35 may participate in the processing and endocyclic recycling of α-synuclein [43]. This proposed role of Rab35 in PD is in part supported by several studies that have shown α-synuclein to interact with other Rab proteins [45, 46]. The level of Rab35 in patient serum may be useful in the diagnoses of different Parkinsonian disorders.

In addition, activation of leucine-rich repeat protein kinase 2 (LRRK2), caused by autosomal dominant missense mutation, predisposes patients to PD [47]. LRRK2 is a Rab GTPase that has been found to phosphorylate 14 Rab proteins, including Rab35 [48]. Specific LRRK2 antibodies that recognize phosphorylated forms of Rab proteins have been developed to examine how LRRK2 and Rab proteins contribute to PD and may serve as a potential therapeutic tool [49, 50].

4.2. Rab35 controls exosome secretion in Down syndrome patients

Early endosomal abnormalities have been correlated to developmental brain defects in Alzheimer's disease (AD) and Down syndrome patients (DS) [51]. In the neurons of these patients, their endosomes are aberrantly numerous and enlarged with accumulated materials that lead to neuronal vulnerability and degeneration [52]. Early endosomes are the first

vesicular compartment along the endocytic pathway where internalized cargos are delivered to late endosomes or multivesicular bodies (MVBs) for sorting to either lysosomes for degradation or to the extracellular space via exosomes release (EVs). The docking of MVBs to the plasma membrane is regulated by Rab35 [6]. In human brain homogenates, Rab35 proteins were at a higher level in DS patients compared to controls using western blotting detection [42]. In addition, DS patients and Ts2 mice (murine model for DS) have higher levels of exosome-enriched EVs and Rab35 in the brain extracellular space [42]. Rab35 is proposed to play a protective role in mediating exosome release to relieve neurons of the toxic materials in neuronal endosomes in DS and AD patients [42].

4.3. Rab35 functions in cell migration and cancers

Rab35 has been shown to interact with effector proteins that are involved in cell adhesion and cell migration which are key processes that are disrupted in cancer. Rab35 is required for Wnt5a/Dvl2-induced Rac1 activation and cell migration in MCF-7 breast cancer cells [5]. Upon *in vitro* Wnt5a stimulation, Dvl2, Rab35 and Rac1 are activated to promote cell migration in MCF-7 breast cancer cells. Conversely, a knockdown of Wnt5a using siRNA resulted in significantly lowered expression of Rab35 and decreased MCF-7 cell migration [5]. Knockdown of Dvl2 also blocked Wnt5a-induced Rab35 and Rac1 activation, supporting that Rab35 was the downstream target of Wnt5a/Dvl2 signaling in MCF-7 breast cancer cells. Rab35 knockdown also resulted in decreased Rac1 activation and cell migration. Further, Rab35 was found to physically associate with Dvl2 in MCF-7 cells using immunofluorescence and co-immunoprecipitation assays. Thus, Wnt5 signaling results in activation of Dvl/Rab35/Rac1 activity that promotes breast cancer cell migration.

In another study, active Rab35 and its effector protein, MICAL1, control cell invasive phenotype in breast cancer cells [53]. MICAL1 has been shown to upregulate reactive oxygen species (ROS) in HeLa cells and phosphorylate proteins leading to malignancies and metastasis. Breast cancer cells receive signals from their microenvironment, such as epidermal growth factor (EGF), LPA and hypoxia, ROS level in cells may increase and functions as second messengers in intracellular signaling cascades to induce their metastasis [54]. Upon stimulation of EGF, Rab35 levels increased in MCF-7 cells [53]. Rab35 knockdown using siRNA in MCF-7 cells showed a dramatic decrease in cell invasion, demonstrating that Rab35 was required for EGF-induced invasion in breast cancer cells [53]. Transfection of cells with siRab35 or siMICAL1 led to decreased ROS, indicating that both Rab35 and MICAL1 are required for ROS generation. Further, the generated ROS was found to activate the PI3K/Akt pathway which also plays a key role in migratory potential regulation. Consistent with this result, knockdown of Rab35 or MICAL1 by RNAi resulted in decreased phosphorylated Akt (P-Akt) [53]. Similarly, P-Akt was higher when MICAL1 or Rab35-GTP (active) were overexpressed in MCF-7 cells. Together, these results revealed that Rab35 and MICAL1 promote ROS production which leads to PI3K/Akt signaling activation, resulting in increased breast cancer cell migration and invasion [53].

Consistent with the role of Rab35 in activating the Akt signaling pathway, an earlier study identified that Rab35 functions downstream of growth factor receptors and upstream of the Akt signaling pathway [55]. Using lentiviruses that express short hairpin RNAs (shRNAs) targeting genes coding for all known G-proteins and lipid/protein, Rab35 knockdown was found

to downregulate Akt phosphorylation [55]. Furthermore, the PI3K-dependent phosphorylation of FOXO1/3A was also decreased in cells depleted of Rab35. Wild type, constitutively active Rab35-Q67L and DN Rab35-S22N was each expressed in cell lines and only the Rab35-Q67L active form bound to and activate FOX01/3A and the P13K/AKT signaling pathway [55]. Based on missense mutations previously identified in the proto-oncogene KRAS in myeloid leukemia patients and colorectal tumors, Rab35 with A151T and F161L mutations were tested for their effect on AKT signaling [56, 57]. Interestingly, Rab35-A151T and Rab35-F161L mutants expressed stably in NIH-3T3 cells also resulted in elevated AKT phosphorylation levels, indicating that these gain-of-function alleles are sufficient to activate PI3K/AKT signaling [55]. Therefore, these studies demonstrated that Rab35 activates AKT signaling in cancer cells to suppress apoptosis and aid in cell transformation.

Rab35 plays an important role in non-small cell lung cancer (NSCLC) cell migration by regulating the interaction of RUSC2 (Rab35 effector protein) and GIT2 (Arf6-GAP) [58]. Both RUSC2 and GITS2 have been found to regulate cell polarity and directional cell migration [16, 59]. The function of RUSC2 is not well characterized and may participate in vesicle-mediated transport and secretory pathway to regulate directional migration [60]. GIT2 interacts with paxillin to mediate normal cell spreading and lamellipodia formation [61]. Upon EGF stimulation, Rab35 is activated and promotes the binding of RUSC2 to the non-phosphorylated form of GIT2 [58]. Knockdown of Rab35 or RUSC2 by RNAi resulted in decreased GIT2 phosphorylation and its half-life, indicating that Rab35 and RUSC2 are each essential for GIT2 phosphorylation and stability [58]. The phosphorylated form of GIT2 is released from RUSC2 and localizes to the plasma membrane to mediate cell migration [58]. Collectively, these data indicate that upon EGF stimulation, active Rab35 promotes the interaction of RUSC2 and GIT2, the intracellular stabilization and phosphorylation of GIT2, and lung cancer cell polarization and cell migration [58].

4.4. Rab35 may regulate T-cell receptor signaling

Upon receptor complex activation, the duration of signaling is affected by alterations in receptor internalization, recycling, and degradation [62]. The prolonged activation of T-cell receptor (TCR) on immune T_H2 cells promotes allergic asthma [63]. TCRs within the plasma membrane of T_H2 cells are dynamically regulated through endocytosis and recycling [64, 65]. The role of Rab35-GEF, DENND1B, in allergic asthma was investigated in mice [63]. The independent knockdown of DENN1B, Rab35, or clathrin adaptor AP-2 resulted in delayed TCR downmodulation after its activation [63]. This in turn resulted in aberrant, prolonged TCR signaling and increased cytokine secretion of IL-4, IL-5, and IL-13 in T_H2 cells. The ability of DENND1B to interact with AP-2 and mediate Rab35 GTP exchange is required for optimal regulation of surface TCR signaling in T_H2 cells [63]. These findings were consistent with enhanced *in vivo* T_H2-mediated inflammation in Dennd1b−/− mice when challenged with aerosolized antigen [63]. The regulation of DENND1B of T_H2 cells provides a potential mechanistic basis to explain the genetic association observe with DENND1B gene variants in early childhood asthma [66, 67].

4.5. Pathogens use the Rab35 pathway during infections

Rab35 has been demonstrated to be involved in the process of erythrophagocytosis of *Entamoeba histolytica* trophozoites, leading to amoebic colitis [68]. *E. histolytica* infect around 50 million people worldwide, and around 100,000 people die annually from this infection [69]. The ability

of *E. histolytica* trophozoites to phagocytose host red blood cells (RBCs), immune cells, apoptotic cells, and microbiota during infection is critical for their pathogenicity [70]. Using an amoebic expression plasmid for *E. histolytica* Rab35 (EhRab35), a previous study found that EhRab35 is mainly localized to both large vacuolar and smaller punctate structures that are distinct from giant early endocytic vacuoles in the cytoplasm [68]. Infection of human RBCs with a stable transgenic line of trophozoites expressing either active Rab35-Q67L or the inactive Rab35-S22N, EhRab35 was found to be involved in the early phase of erythrophagocytosis [68]. Specifically, the active Rab35-Q67L localized to the site of the phagocytic cups during erythrophagocytosis. In addition, live cell imaging data revealed that GFP-EhRab35 translocated to the newly formed actin-based phagocytic cup rapidly from the attachment of RBCs [68]. The number of phagocytic cups decreased when RBCs were incubated with the inactive form of Rab35-S22N trophozoites. Collectively, these results indicate that EhRab35 mediates phagocytosis of RBCs during infection and is involved in phagosome maturation and degradation of RBCs [68].

Uropathogenic *Escherichia coli* (UPEC) are the causative agent of 80% of the urinary tract injections (UTIs) [71]. UPEC hijacks the vesicular trafficking proteins of host bladder epithelial cells (BECs) to facilitate its survival [72]. In comparison to other tested Rab proteins, Rab35 showed a rather striking degree of localization to UPEC-enriched vesicular structures, termed UPEC-containing vacuoles (UCVs) [72]. BECs over-expressing GFP-tagged Rab35 protein were infected with UPEC. Results indicated that Rab35 is recruited to the UPEC-containing vacuoles during intracellular infection of BECs, and Rab35 protein is also increased during UPEC infection [72]. Rab35 knockdown with RNAi in BECs led to a significant reduction in the intracellular bacterial load, demonstrating that Rab35 plays an important role in the intracellular survival of UPEC in bladder epithelial cells. Mechanistically, UPECs usurp the host membrane trafficking system to facilitate trafficking of iron containing (transferrin) vesicles to their residing vacuoles [72]. This is supported by confocal microscopy analysis that showed colocalization of transferrin receptor 1 (TfR1) and Rab35 at the cell surface of BECs during UPEC infection. Both Rab35 and TfR1 knockdown BECs led to reduced iron pool and reduced survival of UPEC. Interestingly, iron supplementation in BECs during UPEC is not able to support intracellular UPEC survival in the absence of host Rab35 or TfR, indicating that the Rab35 pathway is critical for iron acquisition and survival during intracellular UPEC infection [72].

An additional function of the Rab35 recruitment from the host is to promote UPEC survival by preventing the fusion of UPEC-containing vesicles with the hosts' degradative lysosomes [72]. UPECs colocalized with lysosomes in normal and Rab35-deleted cells in late endosomes and lysosomes. In Rab35 knockdown BECs, a significantly higher number of intracellular UPECs colocalized with the lysosomal marker where UPECs are destroyed. Thus, UPECs utilize the host Rab35 mediated vesicular trafficking pathways to enhance its iron acquisition and prevent lysosomal degradation within the bladder epithelial cells during infection [72].

Enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) cause food-poisoning outbreaks, in which patients suffer from diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS) [73]. EHEC is common as a food or water-borne pathogen in industrialized countries, while EPEC remains a significant cause of diarrhea in low-income countries. Another characteristic of EHEC but not EPEC is the production of Shiga toxins, which are associated with the more severe hemorrhagic colitis and HUS [74]. During infections, both EHEC and EPEC deliver bacterial effector proteins into host gut epithelial cells to facilitate

their colonization [75]. One of these bacterial effector protein is EspG, which is critical for EHEC to deplete cell surface receptors from the plasma membrane during infections [76]. EspG may reorganize multiple host signaling networks during infection by binding to Arfs and Rab GTPases [77]. Recently, EHEC EspG during infection was found to modulate an ARF6:Rab35 signaling axis to prevent the host cell's recycling endosome function [78]. EspG binds with Arf6-GTP and not Arf6-GDP in a co-immunoprecipitation study [78]. Interestingly, EspG also acts as a Rab-GAP for Rab35 and other Rabs where it inactivates Rab35. During infections, EspG preferentially interacts with Arf6-GTP upstream of Rab35 binding to transport itself to endosomal structures and prevent Rab35 in mediating recycling of cargo to the host cell surface. Thus, the EHEC secretes its effector protein EspG to shut down the recycling endosomal trafficking in the host cell.

Legionella pneumophila is the causative agent for Legionnaires disease, a community or hospital-acquired pneumonia [79]. *L. pneumophila* proliferates within amoebae in nature and infects human alveolar macrophages. Alveolar macrophages engulf *L. pneumophila* through phagocytosis; however, by secreting over 250 effector proteins that modify the host signaling networks and cell membrane trafficking machineries, this intracellular pathogen converts the macrophage phagosomes into the *Legionella*-containing vacuoles (LCV) where *Legionella* survive and replicate. One of the *L. pneumophila* effector proteins is LepB, which is secreted by *Legionella* at later stages of infection and accumulates in the host cell [80]. In biochemical enzyme kinetic studies, LepB was identified to act as a Rab-GAP to inactivate multiple Rab proteins [80]. LepB can stimulate GTP hydrolysis on Rab35, although with 10–100 times decreased catalytic efficiency than Rab1b [81]. The proposed function of LepB is that it may target multiple Rab proteins that are involved in the biogenesis or maintenance of the LCVs to promote *Legionella* survival.

Another *L. pneumophila* effector protein is AnkX, a phosphocholine transferase that covalently links a phosphocholine moiety to Rab35 [82]. Fluorescent protein-tagged AnkX introduced to mammalian cells was found to localize at the plasma membrane and tubular membrane compartments by transmission electron microscopy [83]. Consistent with its targeting of the endocytic recycling pathway, AnkX co-localized with Rab35 and a Rab35 cargo, the major histocompatibility class I protein (MHCI) [83]. AnkX mediated phosphocholination of Rab35 has been found to stabilize phosphocholinated Rab35-GDP in the membranes [84]. These sequestered membrane-bound phosphocholinated Rab-GDP proteins cannot be converted to the Rab35-GTP form, because they cannot interact with cellular Rab35-GEFs [84]. The phosphocholine modification of Rab35 by AnkX was found to be critical for preventing the activation of Rab35 and prevent the fusion of LCV with phagosomes [83, 85]. Thus, AnkX mediates post-translational modification to Rab35 to manipulate the host endocytic recycling trafficking to evade the immune system.

Rab35 may play a role in the pathogenesis of *Bacillus anthracis* infection. The lethal toxin (LT) is made of protein subunits lethal factor (LF) and protective antigen (PA). PA oligomerizes into ring-shaped structure that bind to four LF subunits that then gets taken up by host cells via clathrin-mediated, dynamin-dependent endocytosis to the early endosomes [86]. The anthrax PA forms a pore into the membrane of the intraluminal vesicles (ILV) that can be delivered to the cytosol or released into the extracellular medium as exosomes which can be taken up by naive recipient cells to propagate infection [87]. Rab11 and Rab35 knockdown in RPE cells failed to trigger MAPKK cleavage in naive RPE-1 cells [7]. Furthermore, Rab35

knockdown prevented exosome formation, indicating that Rab35 is required for the delivery of LF-containing exosomes to extracellular medium in RPE1 cells where these LF-containing exosomes can be taken up by additional cells [7].

5. Conclusions

Rab35 is a highly conserved small GTPase that is the only Rab that mediates endosomal recycling of target proteins between the plasma membrane and the early endosomes. Further understanding of the interactome of Rab35 will elucidate additional functions of Rab35 in the context of development and disease.

Conflict of interest

The authors declare no conflict of interest.

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